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Thermal and pH sensitivity of avian corona and influenza viruses: A model study for inactivation of SARS-CoV-2 (COVID-19) and other flu viruses

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ABSTRACT

Present study has been designed to clarify the mechanism of inactivation of the RNA enveloped viruses *in-vitro*. For that, avian *Coronavirus* (Avian Infectious Bronchitis Virus, AIBV) and Bird flu virus (Low Pathogenic Avian Influenza Virus, LPAIV, H9N2) were selected for this study. Both the viruses were subjected to heat (25 to 70°C and pH (1.0 to 14.0) treatment for 1 to 120 min and 30 s to 5 min respectively and their infectivity was checked by measuring virus titre through hemagglutination (HA) test, chicken embryo inoculation and RT-iiPCR. The results clearly indicates that both the viruses required 55°C for 90 min, 60°C for 60 min, 65°C for 15 min and 70°C for 2 and 3 min respectively to inactivate. Results of pH sensitivity using various detergents and chemicals exposure indicates that the agents having moderate acidic pH (5.0 to 6.0), neutral to moderate alkaline pH (7.0 to 10.0) and extreme alkaline pH (13.0 to 14.0) could inactivate AIBV within 20 to 30 s and the H9N2 virus was inactivated in pH range <3.0 and 13.0 to 14.0 within 5 min. The 70% ethanol or isopropanol was found highly effective to inactivate both the viruses in a minute. More interestingly, juice of various citrus fruits also exhibited antiviral activities *in-vitro*. Results of the present study indicated that the enveloped RNA viruses of any families could easily be inactivated using very cheap and available chemicals and detergents. Chicken embryos propagation and molecular detection by RT-iiPCR also indicated that ultra-acidic and extreme alkaline pH and higher temperature treatment successfully inactivated both the viruses. Finding of the present study also indicated that any viruses of the family *coronaviridae* including SARS-CoV-2 and other respiratory viruses of human and animal can be inactivated easily by exposure at high temperature and extremely low and high pH treatment.

Keywords: Avian infectious bronchitis virus (AIBV), low pathogenic avian influenza virus (H9N2), SARS-CoV-2 (COVID-19), thermal inactivation, pH treatment, chicken embryo inoculation, RT-iiPCR.

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INTRODUCTION

The most important respiratory viruses of human threat belong to the family *Orthomyxoviridae* and *Coronaviridae*. Viruses of the family *Orthomyxoviridae* are single stranded, negative sense, RNA enveloped with segmented (eight genes) genome (ICTV, 2011). The disease caused by viruses of this family is known as influenza. There are three major types of influenza viruses (Type A, B and C) (CDC, 2019). Of the three types, type A viruses are responsible for the production of flu like diseases in wide range of avian species, human

and mammals and divided into 18 sub-classes based on their surface glycoprotein HA (H1-18) and 11 on the basis of NA (N1-11) (CDC, 2019). Many sub-types of influenza viruses which did not possessed strong zoonotic properties yet but is mildly pathogenic for poultry like H9N2 and H5N2. Of the HPAIV, H1N1 and H5N1 are already familiar with everyone because of their high zoonotic characteristic. Of the two sub-types any one can cause mild to severe form of respiratory disease either single or together with other respiratory viruses like human corona viruses of the genus alpha (HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1) or beta (SARS-CoV-1/MERS-CoV/SARS-CoV-2) at any age, sex and group of people at any season of a year globally.

Most records of emerging, re-emerging and trans-boundary infectious diseases of human of the past few decades indicate that around 70% of their infectious diseases appeared as regional and global pandemic threat has been originated from different kinds of wild and domesticated animals (FAO, 2013). Most of the devastating diseases of human were contagious and their causal agents were viruses (HIV-AIDS, Ebola, Dengue, Chikungunya, Japanese encephalitis, West Nile, Zika, Lassa fever, Pandemic bird flu, Swine flu, SARS-CoV-1, MERS-CoV and SARS-CoV-2).

Coronaviruses (CoV) are a group of related viruses that cause diseases in human, mammals and birds. Corona viruses are the largest known RNA viruses, CoVs are further divided into four genera: α-CoVs, β-CoVs, γ-CoVs, and δ-CoVs (Yin and Wunderink, 2018), among which α- and β-CoVs are able to infect human and other mammals, whereas the viruses of other two genera can infect birds as well as mammals (Chen et al., 2020). Out of the four genera of the viruses of the family *Coronaviridae* and subfamily *Orthocoronavirinae* the genus *Gammacoronavirus* (Gamma-CoV) is one of the most important genera of the of the family. They are also positive sense, single-stranded RNA enveloped viruses of zoonotic origin. While the alpha and beta genera are derived from the bat gene pool, the gamma and delta genera are derived from the avian and pig gene pools (Woo et al., 2012). Gamma-CoV also known as coronavirus group 3 (avian coronaviruses). A broad range of coronaviruses are found in bats, which might play a crucial role in the virus evolution of alpha and beta coronavirus lineages in particular. However, other animal species can also act as an intermediate host and animal reservoir (Channappanavar and Perlman, 2017).

SARS is caused by a coronavirus (SARS-CoV) normally found in wild animals such as the palm civet cat and Chinese ferret badger (Donnelly et al., 2004). SARS spread quickly from China to other parts of Asia, Europe, America and elsewhere, infecting more 8000 individuals in 29 countries and killing at least 774 people. Transmission was linked to close contact with cases, mostly in hospital, affecting healthcare workers or patients (Lau et al., 2005; Donnelly et al., 2004). Middle East respiratory syndrome-related coronavirus (MERS-CoV/ EMC/2012/HCoV-EMC/2012), is a species of coronavirus which infects humans, bats and camels (Wong et al., 2019).The infecting virus is an enveloped positive-sense, single-stranded RNA virus which enters its host cell by binding to the DPP4 receptor (Fehr and Perlman, 2015). There were 2494 laboratory confirmed cases and 858 virus related deaths occurred due to MERS-CoV (WHO, 2013).

A deadly viral disease of human known as COVID-19 and its causal agent is the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) has emerged recently as a global pandemic threat originated from the Wuhan city of Hubei province, China in December 2019 (Huang et al., 2020; Chang et al., 2020). As of June 4, 2020, coronavirus COVID-19 is affecting more than 213 countries and territories around the world and 2 international conveyance (the Diamond Princess cruise ship and MS Zaandam cruise ship) having 6,603,144 patients confirmed positive by nucleic acid testing and it has caused 388,502 deaths due to acute respiratory failure or other related complications. WHO declared the outbreak of COVID-19 in China as a Public Health Emergency of International Concern as a pandemic threat globally (WHO, 2020). The first outbreak of COVID-19 in Bangladesh on March 8, 2020 was confirmed three cases by IEDCR, Bangladesh (The Daily Prothom Alo Newspaper). The number of coronavirus infected patients in the country has risen to 57,563 and 781 deaths happened after the disease broke out in China till date. Probably, the diseases came in to Bangladesh from Italy by expatriate people (Worldometer, 2020).

Different types of chemicals, disinfectants and detergents are used for inactivation of virus at different concentration of different pH values for different time. The chemical disinfectants are mostly used for viruses are mostly chemicals including alcohols like ethanol and isopropanol. Alcohols are effective against enveloped viruses such as corona virus, influenza virus and human immunodeficiency virus (HIV) but not small non-enveloped viruses such as parvovirus and poliovirus. Some of the more widely used virus inactivation processes are follows as solvent/detergent inactivation, pasteurization (heating) and acidic/alkaline pH inactivation (Boschetti et al., 2005; Sofer, 2003).

The infectivity of SARS-CoV was lost after heating at 56^{o}C for 15 minutes but that it was stable for at least 2 days following drying on plastic (Lai et al., 2005). SARS-CoV was easily inactivated at 60°C for 60 minutes (Yunoki et al., 2004). The infectivity of MERS CoV was reduced 4 log10 at 56°C for 25 minutes. Increasing temperature to 65°C had a strong negative effect on viral infectivity for 1 minute exposure (Leclercq et al., 2014). All the strains of avian infectious bronchitis viruses were sensitive to heating at 56°C for 15 min. KH and Massachusetts-41 strains of avian infectious bronchitis

viruses were resistant to heating at 45°C for 90 min (Otsuki et al., 1979). LPAI H7N2 viruses were completely inactivated at 56°C after 60 min or at 60°C after 10 min, while retained their infectivity in a water bath at 56°C after 30 min (Castro et al., 1998). A study on AIV (H7N3) indicated that the virus was lost their infectivity at 56°C for 60 min (Muhammad et al., 2001). SARS-CoV was completely inactivated at very strongly alkaline (pH>12.0), or acidic (pH < 3.0) conditions (Pagat et al., 2007; Darnell et al., 2004). All strains of AIBV were resistant to 3.0 but most of the strains were sensitive to pH 11.0 (Otsuki et al., 1979). LPAI (H7N2) virus lost 100% infectivity under pH 2.0 conditions for 5 min (Lu et al., 2003). Another LPAI virus H7N3 was inactivated at pH 1.0, 3.0, 10.0 and 14.0 for 48 hours (Muhammad et al., 2001).

There is no effective treatment and vaccine of the newly pandemic viral disease (COVID-19) yet to stop its devastating activities for human population. Present study has been designed with a view to apply knowledge gathered by determining the sensitivity to the temperature and pH of the two important RNA enveloped viruses (positive and negative senses) of the family *Coronaviridae* (AIBV) and *Orthomyxoviridae* (H9N2, LPAIV) as a model for the SARS-CoV-2 virus.

MATERIALS AND METHODS

Virus samples

Laboratory confirmed, a single stranded positive sense RNA enveloped AIBV of gamma genus of the family *Coronaviridae* and a negative sense, single stranded RNA enveloped virus of the family *Orthomyxoviridae* the H9N2 strain LPAI virus were obtained from the R & D unit of the FnF Pharmaceutical Ltd. Jhenaidah, Bangladesh. These two different species of the purified RNA enveloped viruses were used for this study.

Preparation of 1 and 5% chicken red blood cells (cRBC) suspension

Blood sample was collected aseptically with sterile syringe and needle from the wing vein of sero-negative chicken using 4% tri-sodium citrate solution at a ratio of 1:9. Collected blood was transferred into sterile test tube containing sterile phosphate buffer saline (1X PBS) solution and then centrifuged at 1,500 rpm for 20 min. The supernatant was poured off and the pellets of red blood cells (RBC) were re-suspended and the washing of cRBC was repeated 3 times with PBS by centrifugation. After the final centrifugation, 1 and 5% cRBC suspension was prepared using the 1 and 5 ml pellet of cRBC in 99 ml and 95 ml of 1X PBS, respectively. The suspension was stored at 4°C until use for slide and micro-plate HA tests.

Preparation of robotic chicken cRBC

A ratio of 1:10 sterile tri-sodium citrate was used for the collection of venous blood of a healthy cock. The anti-coagulated cRBC was mixed with sterile 1XPBS and allowed to centrifuge at 1500 rpm for about 20 min and the supernatant was discarded. The cRBC was washed for three times following the previous one. After final washing the pellet of cRBC was added with sterile 1XPBS at a concentration of 1% cRBC after that 1% of 37% commercial formaldehyde was added. Formaldehyde added cRBC was allowed to stirrer at 8 to 10 rpm under refrigerated condition for a period of 4 days after that the cRBC was allowed to centrifuge at 2,000 rpm for 20 min and the formalin containing supernatant was discarded and the washing process was repeated for three times and the prepared cRBC was tested for possessing its resistant capacity against hypotonic (normal water) and hypertonic solution (concentrated sodium chloride solution). The prepared cRBC was called robotic cRBC because this RBC can capture any kind of hemagglutinating RNA viruses from lake, river or sea water. Once the robotic cRBC preparation is ready this cRBC could be used several months keeping at 4 to 8°C and even under lyophilized condition for several years.

Determination of infectivity of the enveloped RNA viruses by heat treatment

The avian infectious bronchitis virus (AIBV) and H9N2 viruses were subjected to inactivate at various temperatures under control condition (Digital rotary water bath, B206, FIRSTEK, Taiwan). The effect of heat treatments for the inactivation of both strains of viruses was determined by slide and plate HA test using 5 and 1% robotic cRBC and normal cRBC. For thermal inactivation of 1024 and 8 HA units of AIBV and H9N2 viruses were determined by micro-plate HA test. 1 ml of 1XPBS containing 1024 and 8 HA units of each of the two viruses (AIBV and H9N2) were added into thin-walled 1.5 ml sterile eppendrof tube (Axygen scientific, USA) and placed in the digital hot-water bath setting at different temperatures, from 25, 30, 37, 40, 45, 50, 55 and 60°C for every 30 min intervals up to 120 min (Table 1), for 65 and 70°C at 1 minute interval for 15 min (Table 2).

Determination of infectivity of the enveloped RNA viruses after exposure to different detergents, inorganic and organic chemicals of variable pH

The infectivity of the AIBV and H9N2 viruses under treatment with different pH (acidic and alkaline) at different time intervals, the viruses were exposed to different types of chemicals (Rok Bleaching powder, Hexisol, Ethanol, $NaHCO_3$, Hydrogen peroxide Mega-C Injection, Ceevit Tablet, Cider vinegar, Acetic Acid and Citric acid), disinfectants and detergents (Wheel Laundry soap, Surf Excel powder, Lizol floor wash, Savlon floor wash, Dettol floor wash, Vixsol floor wash, Vim liquid dishwashing, Trix liquid dishwashing, Glitter liquid dishwashing and Clean Max), Toiletries (Sepnil liquid hand wash, Savlon liquid hand wash, Dettol liquid hand wash, Lifebuoy liquid hand wash, Sepnil hand sanitizer and Savlon bath soap), citrus food (30% Lemon Juice, 50% and 100% Orange juice, 100% Orange peel, 50% and 100% Malta juice) and different types of ashes (cow dung ash, bean leaf ash, rice husk ash, banana leaf ash and rain tree ashes) at different concentration of different pH values (3.0 to 14.0) for different time intervals at room temperature. The substances (chemicals, disinfectants and detergents, Toiletries, citrus food and different types of ashes) were categorized according to pH (Soil survey manual, 1993) as ultra-acidic (<3.5), extremely acidic (3.5 to 4.4), strongly acidic (5.1 to 5.5), moderately acidic (5.6 to 6.0), slightly acidic (6.1 to 6.5), neutral (6.6 to 7.3), slightly alkaline (7.4 to 7.8), moderately alkaline (7.9 to 8.4), strongly alkaline (8.5 to 9.0) and very strongly alkaline (>9.0) (Tables 3 and 4). 1 ml virus solutions containing two different concentration of HA titre, 1024 and 8 HA of each of the two viruses (AIBV and H9N2) were treated at different concentration of the above mentioned substances having pH

Table 1. Effect of heat on membrane glycoproteins, viability of the viruses and their genome after exposure at the temperatures 25-60°C.

Type of viruses	Heat treatment (°C)	Detection of activity of membrane glycoproteins of the viruses by HA test								No. of death and survived chicken embryos								Genome detection by RT-iiPCR							
		30 min		60 min		90 min		120 min		30 min		60 min		90 min		120 min		30 min		60 min		90 min		120 min	
		H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L
AIBV	25	+	+	+	+	+	+	+	+	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	+	+	+	+	+	+	+	+
	30	+	+	+	+	+	+	+	+	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	+	+	+	+	+	+	+	+
	37	+	+	+	+	+	+	+	+	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	+	+	+	+	+	+	+	+
	40	+	+	+	+	+	+	+	+	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	+	+	+	+	+	+	+	+
	45	+	+	+	+	+	+	+	+	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	+	+	+	+	+	+	+	+
	50	+	+	+	+	+	+	+	+	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/0$	$5^n/0$	$5^n/0$	$5^n/0$	+	+	+	+	+	+	+	+
	55	+	+	+	+	-	-	-	-	$5^n/5^d$	$5^n/5^d$	$5^n/0$	$5^n/0$	$5^n/0$	$5^n/0$	$5^n/0$	$5^n/0$	+	+	+	+	+	+	+	+
	60	+	+	-	-	-	-	-	-	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	+	+	+	+	+	+	+	+
H9N2	25	+	+	+	+	+	+	+	+	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	+	+	+	+	+	+	+	+
	30	+	+	+	+	+	+	+	+	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	+	+	+	+	+	+	+	+
	37	+	+	+	+	+	+	+	+	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	+	+	+	+	+	+	+	+
	40	+	+	+	+	+	+	+	+	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	+	+	+	+	+	+	+	+
	45	+	+	+	+	+	+	+	+	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/0$	$5^n/0$	$5^n/0$	$5^n/0$	+	+	+	+	+	+	+	+
	50	+	+	+	+	+	+	+	+	$5^n/5^d$	$5^n/5^d$	$5^n/0$	$5^n/0$	$5^n/0$	$5^n/0$	$5^n/0$	$5^n/0$	+	+	+	+	+	+	+	+
	55	+	+	+	+	-	-	-	-	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	+	+	+	+	+	+	+	+
	60	+	+	-	-	-	-	-	-	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	$5^n/5^d$	+	+	+	+	+	+	+	+

+ indicates membrane glycoprotein of viruses were found active and – indicates membrane glycoprotein of viruses were not found active by HA test of the viruses of both the H (high, 2048 HA) and L (low, 8 HA) titre used for the test;
No. of the embryos inoculated with the heat treated viruses (n= 5); no of embryos death due to live viruses (d= 5);
In RT-iiPCR, + indicates positive for genome detection, - indicates negative for genome detection.

ranged from 3.0 to 14.0 for 5 min at 30 s intervals. The treated virus suspensions were further tested to determine their hemagglutinating properties with cRBC by micro-plate HA test and RT-iiPCR.

Determination of infectivity of both the viruses through propagation in chicken embryo inoculation

The heat and pH treated viruses were placed in the ice bath immediately and a total of 200 µl heat and pH treated virus suspension containing antibiotics (penicillin 10000 IU + streptomycin 1000 µg/ml) was inoculated into 9-day-old embryo of SPF chicken eggs (CP Bangladesh Co. Ltd.) through allantoic sac route of inoculation with each virus. Five embryos were inoculated for each virus. The remaining heat and pH treated virus suspension was used for HA test and extraction of viral RNA by ready to go GN method for Reverse Transcription-Insulated Isothermal PCR (RT-iiPCR).

Determination of titre of the heat and pH treated RNA enveloped viruses (AIBV and H9N2) by HA test

HA test was performed in a V-bottom 96 micro-well plate to determine HA units (4HA/50µl). This was carried out by two-fold serial dilutions of the viral suspension in a micro-well plate and then tested to determine an end point. For this purpose, 50 µl of PBS was dispensed into each well of the micro-well plate. Then 50 µl of test sample (virus) were placed in first well of each row of column 1 and then two-fold dilution was made up to column 11. A 100 µl of 1% cRBC were added to each well including wells of column 12. The control wells contain only PBS and red blood cells. The plate was allowed to stand for 45 minutes in the refrigerator at 4°C. The results of the plates were read and recorded according to Reed and Muench (1938) method. In HA negative case, a sharp buttoning of red blood cells at the bottom of the V-bottom well and in HA positive case, button of red blood cells at the bottom of the V-bottom

Table 2. Effect of heat on membrane glycoproteins, virus multiplication and genome detection after exposure at 65 and 70°C temperature.

Tests performed	RNA enveloped viruses	Heat treatment (°C)	Activity of the viruses at different time intervals (minutes)																													
			1 min		2 min		3 min		4 min		5 min		6 min		7 min		8 min		9 min		10 min		11 min		12 min		13 min		14 min		15 min	
			H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L
Micro-plate HA test	AIBV	65	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
		70	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H9N2	65	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	
		70	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mean death and survivability of chicken embryo	AIBV	65	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/0	5^n/0
		70	5^n/5^d	5^n/5^d	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0
	H9N2	65	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/0	5^n/0
		70	5^n/5^d	5^n/5^d	5^n/5^d	5^n/5^d	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0	5^n/0
RT-iiPCR	AIBV	65	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
		70	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	H9N2	65	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
		70	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

+ indicates positive activity of the membrane glycoprotein of viruses ; – indicates negative activity of the membrane glycoprotein of the viruses by HA test having H (high, 2048 HA) and L (low, 8 HA) titre used for the test;
n= 5 indicates no. of the embryos inoculated; d= 5 indicates no of embryos death and the titer of the viruses in the allantoic fluid (AF) by HA test
++ indicates genome detection positive by RT-iiPCR.

wells were showed in micro well plate. The HA titre of the virus was determined as the highest dilution of the virus which agglutinates the cRBC in the micro-plate HA test. 4HA titre of the virus was calculated from the 1HA.

Determination of inactivation of the viruses after treatment with different chemical, disinfectant and detergent from the contaminated surfaces

The anti-viral effect of standard concentration of various chemicals, disinfectant and detergents to destroy the RNA enveloped viruses of both the high and low titre were spelled over on the floor of hospital, university library, different laboratories, conference room, government and non-government office, community center and public toilet for a period of 10 min to 1 h. Each floor was subjected to sweep with 1% Rok Bleaching powder and 5% Lizol. After sweeping with the bleaching solution and Lizol each surface of the floor was swabbed and the swab was collected at 1 min, 5 min and 15 min (Table 5). The swabs suspensions were also inoculating into plate count agar media and incubated at 37°C for 24 h. The suspensions were further tested for viruses by using HA test. In this study, two types of ash (10% rich husk ash and 10% banana leaf ash) containing soil pot were kept in the hospital for collecting patient's nasal discharge and cough at 1 min, 5 min and 15 min intervals. After collecting, the discharge was diluted with PBS. The suspensions were also inoculating into plate count agar media and incubated at 37°C for 24 h to check bacterial contamination and further tested for viruses by using HA test.

Preparation of hand sanitizer and determination of its efficacy

Hand sanitizer was prepared using ethanol (grain alcohol, most commonly available at 90 to 95%), glycerol and water. Primarily, 70 ml ethanol was mixed with 10 ml of glycerol and 20 ml of distilled water (WHO, 2020). All the three

Table 3. pH sensitivity of the AIBV after treatment with different detergents and chemicals at different time intervals.

Sl No.	Item name	Concentration	Category of pH	Cellular receptor binding ability of the viruses after treatment with different pH at different time intervals by HA test (every 30 s intervals for 5 min)																			
				30 s		1 min		1 min 30 s		2 min		2 min 30 s		3 min		3 min 30 s		4 min		4 min 30 s		5 min	
				H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L
1	Citric acid	10%	Ultra acidic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	Acetic Acid	5%,10%	Extremely acidic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	Clean Max	5%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	Sepnil liquid hand wash	10%	Strongly acidic	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
5	Dettol liquid hand wash	10%		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	Vixsol floor wash	5%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	Citrus fruits	30%, 50%,100%	Moderately acidic	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	Cider vinegar	5%,10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	Rice husk ash	10%		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
10	Ethanol	70%	Slightly acidic	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
11	Ceevit Tablet	2.5 %		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	Sepnil hand sanitizer	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	Mega -C Injection	10%		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	Vim liquid dishwashing	5%	Neutral	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
15	Trix liquid dishwashing	5%		++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
16	Glitter liquid dishwashing	5%		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	Hexisol	100%	Slightly alkaline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	Hydrogen peroxide (6%)	1%, 10%, 100%		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19	Savlon floor wash	5%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	Savlon bath soap	10%	Moderately alkaline	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
21	Lizol floor wash	5%		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
22	Cow dung ash	10%		++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

Table 3. Continues.

23	Banana leaf ash	10%		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
24	Savlon liquid hand wash	10%	Strongly alkaline	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
25	Lifebuoy liquid handwash	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	$NaHCO_3$	2%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	Dettol floor wash	5%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	Bean Leaf ash	10%	Very strongly alkaline	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
29	Surf Excel powder	10%		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	Raintree wood ash	10%		++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
31	Rok Bleaching powder	1%		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
32	Wheel Laundry soap	1%		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

H indicates high (2048 HA) and L indicates low (8 HA) titre of viruses used for the test; +++ indicate= highly sensitive, ++ = moderately sensitive, +=Mild sensitive and – indicate= not sensitive.

Table 4. pH sensitivity of the H9N2 viruses after treatment with different detergents and chemicals at different time intervals.

Sl No.	Item name	Concentration	Category of pH	Cellular receptor binding ability of the viruses after treatment with different pH at different time intervals by HA test (every 30 s intervals for 5 min)																			
				30 s		1min		1 min 30 s		2 min		2 min 30 s		3 min		3 min 30 s		4 min		4 min 30 s		5 min	
				H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L
1	Citric acid	10%	Ultra acidic	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2	Acetic Acid	5%,10%	Extremely acidic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	Clean Max	5%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	Sepnil liquid hand wash	10%	Strongly acidic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	Dettol liquid hand wash	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	Vixsol floor wash	5%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	Citrus fruits	30%, 50%,100%	Moderately acidic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	Cider vinegar	5%,10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	Rice husk ash	10%		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
10	Ethanol	70%	Slightly acidic	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
11	Ceevit Tablet	2.5 %		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4. Continues.

12	Sepnil hand sanitizer	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	Mega -C Injection	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	Vim liquid dishwashing	5%	Neutral	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	Trix liquid dishwashing	5%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	Glitter liquid dishwashing	5%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	Hexisol	100%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	Hydrogen peroxide (6%)	1%, 10%, 100%	Slightly alkaline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	Savlon floor wash	5%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	Savlon bath soap	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	Lizol floor wash	5%	Moderately	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	Cow dung ash	10%	alkaline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	Banana leaf ash	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	Savlon liquid hand wash	10%	Strongly alkaline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	Lifebuoy liquid handwash	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	$NaHCO_3$	2%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	Dettol floor wash	5%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	Bean Leaf ash	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	Surf Excel powder	10%	Very strongly alkaline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	Raintree wood ash	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	Rok Bleaching powder	1%		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
32	Wheel Laundry soap	1%		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

H indicates high titre (2048 HA) and L indicates low titre (8 HA) of the viruses used for the test; +++ indicate= highly sensitive, ++ = moderately sensitive, +=Mild sensitive and – indicate= not sensitive.

ingredients were mixed thoroughly to make a homogenous mixture. 1 ml virus solution containing two different concentration of each viruses (AIBV and H9N2) having HA titre 1024 and 8 were treated with newly prepared hand sanitizer for 30 s intervals up to 5 min at room temperature for checking the efficacy of the newly made hand sanitizer. The treated virus suspensions were further tested for HA activities by micro-plate HA test.

Molecular detection of RNA of the enveloped viruses (AIBV and H9N2) by rapid RT-iiPCR assay

The infectivity of the heat treated RNA enveloped viruses (AIBV and H9N2) was determined by genomic RNA detection by RT-iiPCR. The AIBV and influenza RT-iiPCR reagent kits (POCKIT AIBV Reagent Set and POCKIT for H9 influenza Reagent Set, respectively) were used to detect the AIBV and influenza viruses. Briefly, the lyophilized premix was rehydrated with 60 µl Premix Buffer B before a 5 µl nucleic acid sample was added. Subsequently, 60 µl of the final mixture was transferred into an R-tube, which was spin briefly in a mini-centrifuge (Mini-centrifuge Type TC10, Hitachi Koki Co. Ltd, Tokyo, Japan) and placed into a POCKIT device. The turnaround time was less than 1 hour. With the programme of POCKIT, the results were shown as '+' (positive) or '–' (negative). For each run, both positive (manufacturer supplied) and negative (PCR-grade water) template controls were used (Figure 1).

RESULTS

Results of thermal sensitivity of the enveloped RNA viruses

The results of thermal treated AIBV and H9N2 viruses of either high (1024) and low (8) HA titre exposed at 25, 30, 37, 40, 45 and 50 for 120

Table 5. Practical approaches of decontamination of different surfaces after contamination with AIBV.

Surfaces	Concentration of disinfectants	Manifestation of HA activity of the swabs samples		
		1 min	5 min	15 min
Hospital floor	Rok Bleaching powder (1%)	-	-	-
University library		-	-	-
University laboratories		-	-	-
University conference room		-	-	-
Government office conference room floor		-	-	-
Community center floor		-	-	-
Public toilet floor		-	-	-
Hospital floor	Lyzol floor wash (5%)	-	-	-
University library		-	-	-
University laboratories		-	-	-
University conference room		-	-	-
Government office conference room floor		-	-	-
Community center floor		-	-	-
Public toilet floor		-	-	-

- indicates failure of showing HA activity of the live virus through binding with the receptor of 1% robotic cRBC.

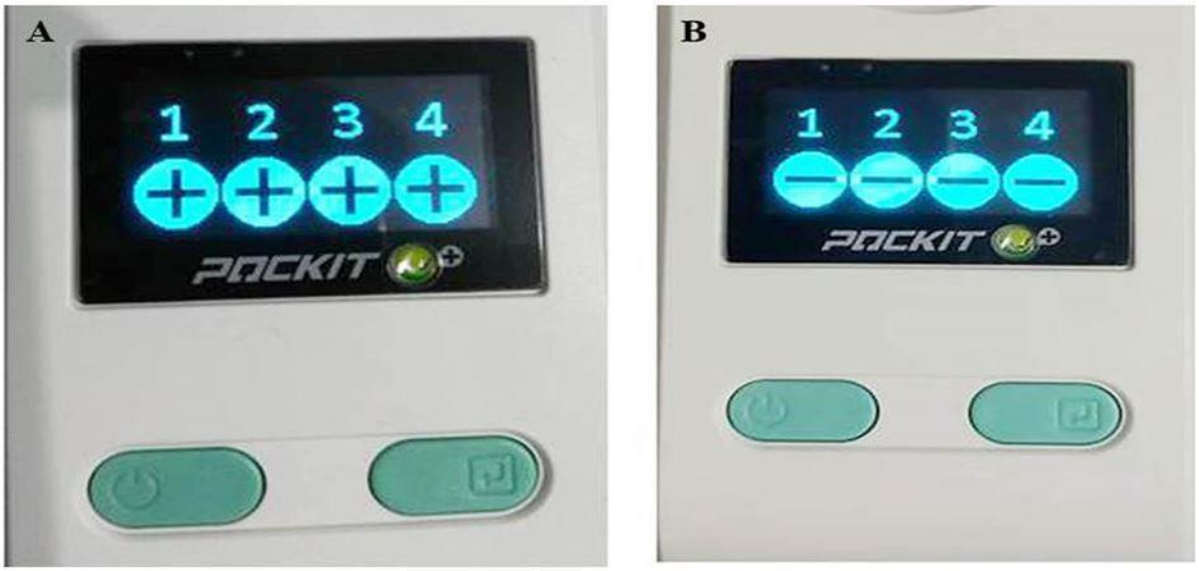

Figure 1. A & B: Results of RT-iiPCR of high pH treated AIBV and H9N2 viruses; A) Lane 1-2 indicate PCR positive for pH-non treated AIBV and (1024 and 8 HA titre); Lane 3-4 indicate PCR positive for pH-non treated H9N2 virus (1024 and 8 HA titre); B) Lane 1-2 indicate PCR negative for pH-treated AIBV (1024 and 8 HA titre); Lane 3-4 indicate PCR negative for pH-treated H9N2 virus (1024 and 8 HA titre).

min were found remained active at these treatment conditions but at 55°C for 90 min and 60°C for 60 min exposure it indicated that both the viruses were found to be inactive determined by slide HA test. The recovery rate of the thermal treated viruses (AIBV and H9N2) when exposed at 25, 30, 37, 40, 45 and 50°C for maximum at 120 min at 30 min interval indicated that both the viruses were remained active but treated at 55°C for 90 min and 60°C for 60 min clearly indicated that the viruses become inactive which was confirmed by chicken embryo inoculation (Table 1). On the contrary, results of the thermal treated viruses (AIBV and H9N2) showed the genomic RNA of both the viruses were remained active when exposed at 25, 30, 37, 40, 45, 50,

55 and 60°C for 120 min at 30 min intervals by RT-iiPCR (Table 1).

Interestingly the results of heat treated AIBV and H9N2 viruses of high (1024) and low (8) HA titre when exposed at 65 and 70°C for a total of 15 min at 1 min interval for a total of 15 min indicated that both the viruses were found inactivated completely at 65°C in 15 min and 70°C in 2 to 3 min respectively by slide and micro-plate HA test (Figures 2 and 3). The inactivation of viruses (AIBV and H9N2) when exposed at 65°C for 15 min and at 70°C for 2 min in case of AIBV and 3 min in case of H9N2 by chicken embryo inoculation. The viruses when exposed at 65°C and 70°C for 15 min and 2-3 min respectively failed to multiply into 9-days-old chicken embryos whereas, the non-heat treated samples containing live viruses were found to multiply well into the chicken embryos and the allantoic fluid (AF) containing viruses were detected by both the slide and micro-plate HA test. The results of the thermal treated (65 and 70°C every 1 min interval for 15 min) viruses showed positivity in genome detection by RT-iiPCR (Table 2).

Results of pH sensitivity of the enveloped RNA viruses by using different chemicals, disinfectants and detergents

The pH sensitivity of the enveloped RNA viruses (AIBV and H9N2) of both the high (1024) and low (8) HA titre were exposed to different pH condition at 30 s intervals for 5 min. The results were described according to pH value of the substances. Treatment at ultra-acidic pH (< 3.5), the AIBV was found not at all sensitive but H9N2 viruses were found highly sensitive to the inorganic 10% citric acid for 5 min exposure determined by micro-plate HA test. Treatment at extremely acidic pH (3.5 to 4.4), the AIBV and H9N2 viruses were found not at all sensitive to the acetic acid (5 and 10%) and 5% clean max for 5 min exposure determined by micro-plate HA test (Figure 4). Treatment at strongly acidic pH (5.1 to 5.5), the AIBV was found highly sensitive except H9N2 to the 10% sepnil liquid hand wash after 30 seconds of exposure. The AIBV was also found moderately sensitive to the 10% Dettol liquid handwash for 30 s treatment. Viruses were found not at all sensitive to the 5% Vixsol floor wash after 5 min exposure. Treatment at moderately acidic pH (5.6 to 6.0), the AIBV was found mildly sensitive at moderately acidic pH but the H9N2 virus was not equally sensitive to the 30% fresh Lemon, Orange (50 and 100%) and Malta (50 and 100%) juice for 30 seconds treatment. On the other hand both the viruses were found resistant to the cider vinegar treatment at 5 and 10% con. Whereas, the AIBV and H9N2 were found highly sensitive to 10% rice husk ash treatment for 30 s (Figure 4). Treatment at slightly acidic pH (6.1 to 6.5), both the AIBV and H9N2 viruses were found highly sensitive when exposed to 70% ethanol and isopropanol for 1 to 2 min (Figure 5). Whereas, the AIBV was found mildly sensitive to 100% orange peel and 2.5% Ceevit Tablet after exposure for 2 to 5 min. Treatment at neutral pH (6.6 to 7.3), the AIBV and H9N2 viruses were found not at all sensitive to the 10% sepnil hand sanitizer even for 5 min of exposure. On the other hand the AIBV was found mildly sensitive to the 10% Mega-C Injection and 5% Glitter liquid dishwashing whereas, for 5% vim liquid dishwashing and 5% Trix liquid dishwashing the virus was found moderately sensitive for 5 minutes of exposure. Treatment at slightly alkaline pH (7.4 to 7.8), both the AIBV and H9N2 viruses was found non-sensitive to 100% Hexisol and 6% Hydrogen peroxide, 5% savlon liquid used for floor sweeping even after 5 min of exposure. Treatment at moderately alkaline pH (7.9 to 8.4), the AIBV was found moderately sensitive but H9N2 virus not at all sensitive to 10% savlon bath soap and 10% cow dung for 30 seconds of exposure. Only the AIBV not the H9N2 virus was found highly sensitive to 5% Lyzol floor wash and 10% banana leaf ash even for 30 s of exposure. Treatment at strongly alkaline pH (8.5 to 9.0), the AIBV was found highly sensitive to 10% savlon liquid handwash, whereas the both viruses were found not at all sensitive to 10% Lifebuoy liquid hand wash during 30 seconds of exposure means 30 s of hand washing. Treatment at very strongly alkaline pH (>9.0), both the AIBV and H9N2 viruses were found highly sensitive to the 1% Rok Bleaching powder and 1% Wheel Laundry soap (Figure 5). The AIBV was found moderately sensitive to the 10% Banana leaf ash, 10% Raintree wood ash and 10% Surf Excel powder and mildly sensitive to 4% $NaHCO_3$ during 30 s of exposure (Tables 3 and 4).

Practical approaches of pH sensitivity of the viruses during surface decontamination

The clear plate count agar is the indication of no growth of microorganisms (bacteria). Buttoning of cRBC in the micro-plate HA test is the indication no viable viruses in the swabs samples of the floor surface collected after one, five and 15 min intervals of sweeping with 1% Rok Bleaching powder, 5% Lizol, 10% rice husk ash and 10% banana leaf ashes. The efficiency of the Rok Bleaching powder, Lizol, rice husk and banana leaf ashes were found 100% effective to inactivate both the bacteria and viruses after sweeping of the different floor surfaces or other solid surfaces (Table 5).

Efficacy of hand sanitizer for the inactivation of the RNA viruses

Efficacy of the home made hand sanitizer was found highly effective for the inactivation of both the RNA

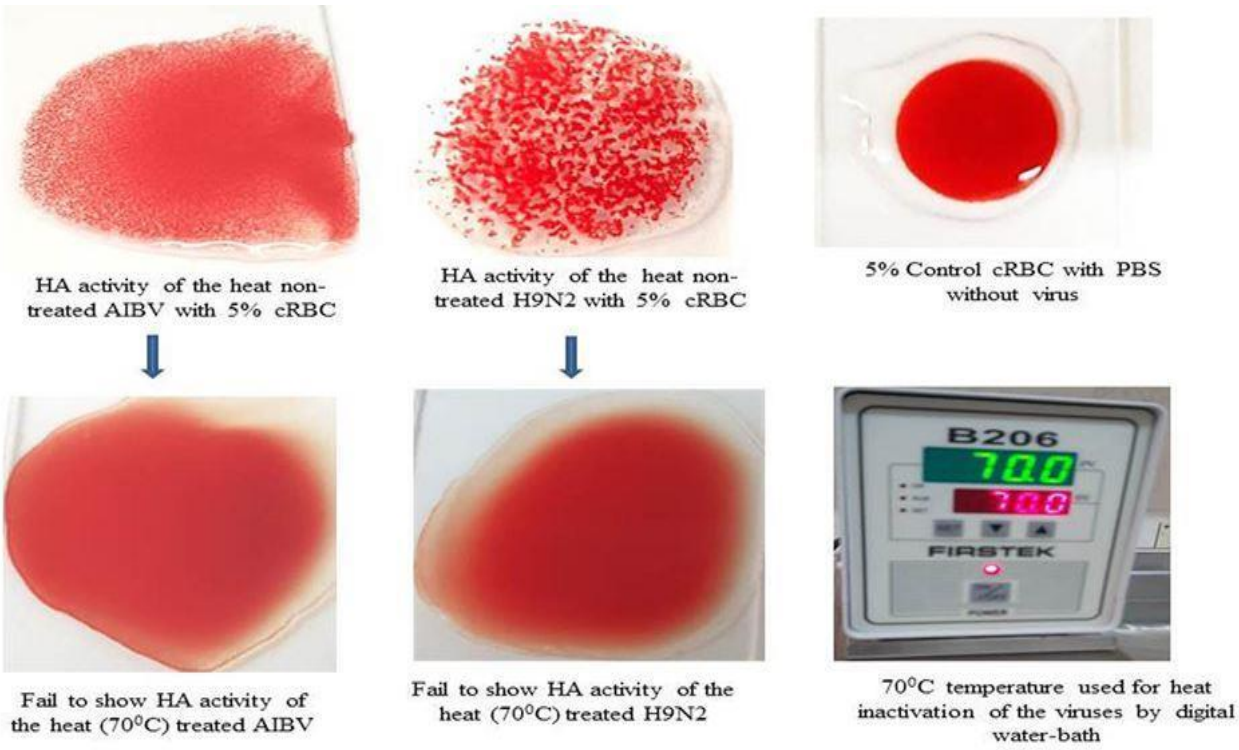

Figure 2. Slide HA activity of the heat non-treated and treated AIBV and H9N2 viruses having 1024 HA titre.

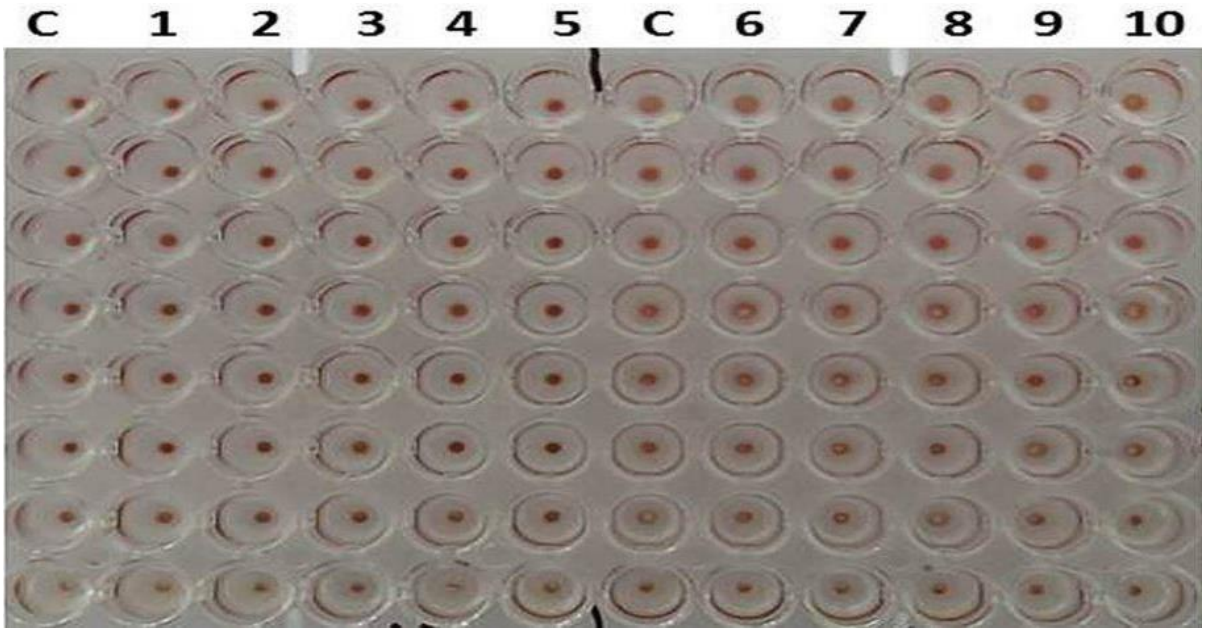

Figure 3. HA activity of the AIBV and H9N2 after heat treatment at 70°C by micro-plate HA test; Colum C indicate buttoning of 1% normal cRBC; Colum 1-5 indicates buttoning of normal cRBC after 2, 3, 4, 5 and 6 min exposure of the AIBV at 70°C; Colum C indicate buttoning of 1% control cRBC; Column 6-10 indicate buttoning of cRBC after 3, 4, 5, 6 and 7 min exposure of the H9N2 at 70°C.

enveloped (AIBV and H9N2) viruses used in this study. Results of both the slide and micro-plate HA test also showed complete inactivation of the viruses after 30 s of exposure.

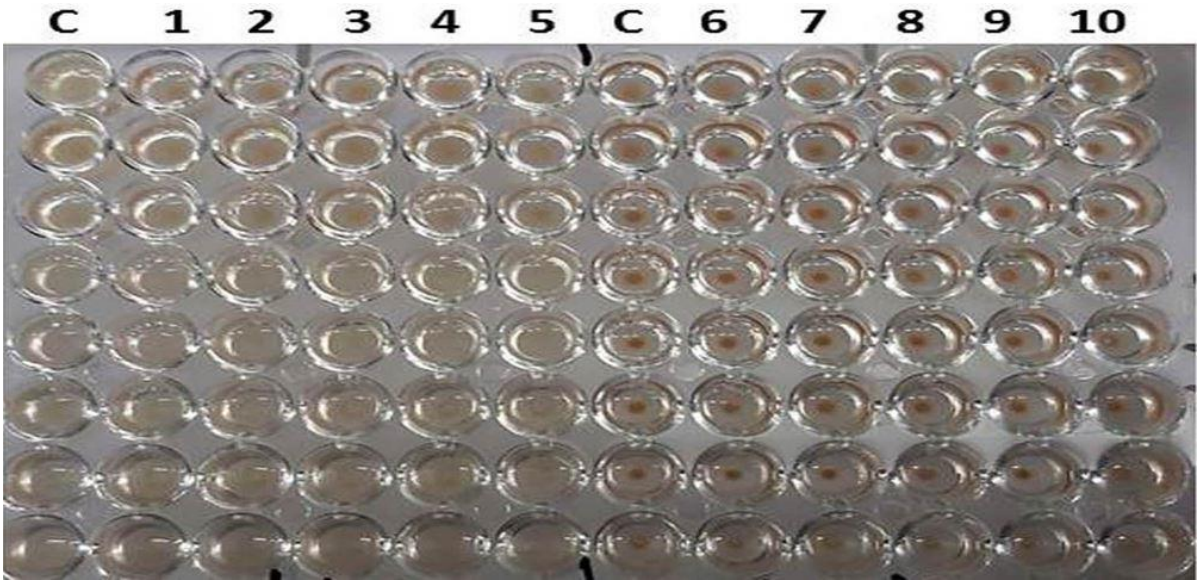

Figure 4. HA activity of the AIBV after treatment with acetic acid and rice husk ash by micro-plate HA test; Column C indicate control AIBV with no buttoning with 1% normal cRBC; Column 1-5 indicates no buttoning of cRBC at 1-5 min of exposure of the virus with 10% acetic acid; Column C indicate buttoning of 1% control cRBC, Column 6-10 indicates buttoning of cRBC at 1-5 min of exposure of the virus with 10% rice husk.

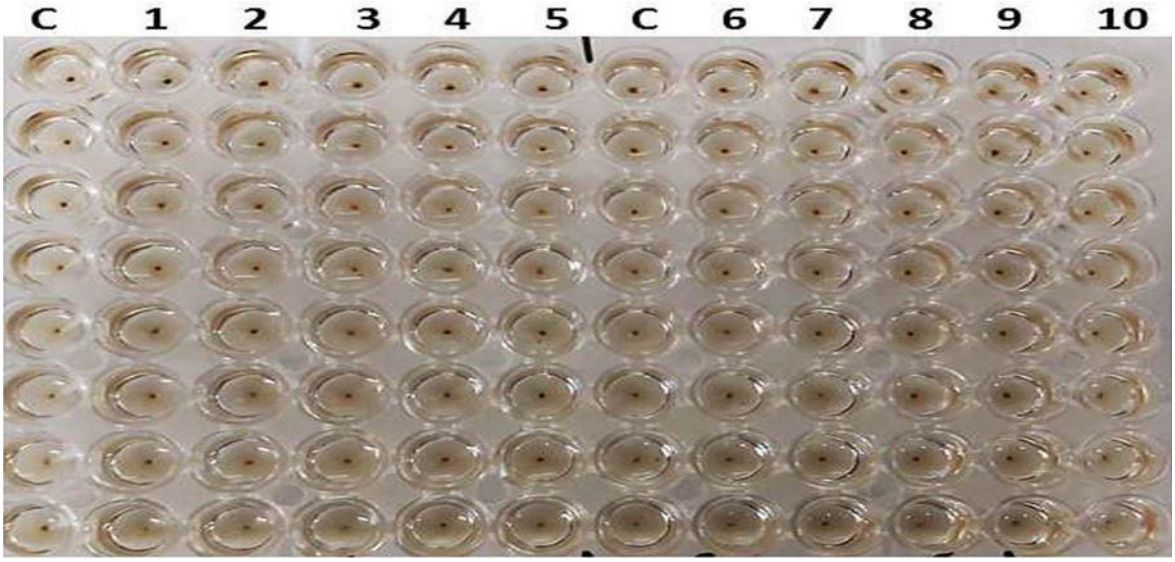

Figure 5. HA activity of the AIBV after exposure with 70% Ethanol and 1% Bleach by micro-plate HA test; Column C indicate buttoning of 1% control robotic cRBC; Column 1-5 indicate buttoning of rcRBC after 1-5 min of exposure of the virus with 70% ethanol; Column C indicate buttoning of 1% control robotic cRBC; Column 6-10 indicate buttoning of rcRBC after 1-5 min of exposure of the virus with 1% bleach.

DISCUSSION

The present study has been designed with a view to determine the effect of heat and pH on the infectivity of the two different species of enveloped RNA viruses using commonly available substances (chemicals, disinfectants, detergents, toiletries, citrus food and different types of ashes) as a model for the inactivation of the circulating COVID-19 virus and other flu viruses which are responsible for mild to severe form of respiratory diseases in human and animals. The partial and complete inactivation of the two viruses by heat and pH treatment in various times of exposure was determined by HA test, virus multiplication in chicken

embryos and genome detection by RT-iiPCR.

The results of thermal sensitivity of the present study indicated that AIBV and H9N2 were effectively inactivated when exposed to high temperature at 55°C for 90 min, 60°C for 60 min and 65°C for 15 min but in 70°C for AIBV in 2 min and for H9N2 in 3 min determined by HA test this might be due to denaturation of membrane glycoprotein receptor like hemagglutinating-neuraminidase (H9N2) and spike protein (SARS-CoV-1, MERS-CoV and SARS-CoV-2) in the envelope of both viruses. Both the viruses failed to multiply in chicken embryos this might be due to failure of attachment and fusion between the viral envelope and cellular membrane which ultimately helps in transferring viral nucleic acid into the cells cytoplasm of the embryos for the multiplication of the virus particle. Though, the RT-iiPCR showed positive result in genome detection of both the heat inactivated viruses. On the contrary, the AIBV and H9N2 viruses multiplied successfully in the chicken embryos even after exposure in variable temperature ranged from 25 to 50°C from 30 to 120 min and also showed HA activity by the AF of the dead embryos in micro-plate and slide HA test and genome detection by RT-iiPCR. The findings of heat treatment of the present study partially supports to the finding of other researchers. All strains of AIBV were found sensitive to heating at 56°C for 15 min (Jackwood et al., 2010; Cavanagh and Gelb, 2008). The infectivity of human corona virus (SARS CoV-1) was found to loss it's infectivity after heating at 56°C for 15 min (Lai et al., 2005). A study of Yunoki et al. (2004) stated that the SARS-CoV-1 could easily be inactivated by the heat treatment at 60°C for 60 min in all in-processed samples. Variable result on heat treatment was also noticed in case of avian influenza viruses. Lu et al. (2003) found that LPAI (H7N2) viruses were completely inactivated after 60 min at 56°C or at 60°C after 10 min, while retained their infectivity in a water bath at 56°C after 30 min. Another study on AIV (H7N3) indicated that the virus remained infectious after being treated at 56°C treatment for 30 min, but its infectivity was lost after 60 min of treatment (Muhammad et al., 2001). The high pathogenic avian influenza (HPAI) H5N1 virus was inactivated after exposure at 56°C for 30 min (Shahid et al., 2009). H5N1 viruses were completely inactivated at 70°C for 60 min or at 75°C for 45 min (Wanaratana et al., 2009).

The results of pH sensitivity of the AIBV after treatment with various detergents, disinfectant and chemicals in different time exposure indicates that those detergent, chemicals and disinfectant having moderate acidic pH (5.0 to 6.0), neutral to moderate alkaline pH (7.0 to 10.0) and very strong alkaline pH (13.0 to 14.0) were found highly effective to inactivate the AIBV and H9N2 viruses surface glycoprotein (S and HN proteins) responsible for binding of host cells receptors within 20 to 30 s this might be due to complete denaturation of the viral enveloped glycoproteins (hemagglutinin neuraminidase and spike protein) of both the viruses which ultimately failed to bind with the cellular receptors (alph-2-3 sialic acid and ACE2 of the H9N2 and AIBV respectively). Chemical treatment of the RNA enveloped viruses not only destroys the viral envelope but also the viral nucleic acid as well. The chemicals and detergents having slightly acidic (6.5) to moderate alkaline pH (7.0 to 10.0) and very strongly alkaline pH (10.0 to 14.0) have found moderately sensitivity to inactivate the AIBV by micro-plate HA test this might be due to mildly denaturation of the viral enveloped glycoproteins proteins which might be the leading cause of failure of binding of the viruses with the cellular receptors completely. On the other hand, the chemicals and detergents having strongly acidic (<3.0) to moderately acidic pH (5.0 to 6.0) and neutral to moderate alkaline pH (7.0 to 10.0) were found mildly sensitive to AIBV by micro-plate HA test. Showing partial HA activity of the virus clearly indicate that there might be some partial denaturation of the whole viral or the amino acids of the viral enveloped glycoproteins which ultimately failed to bind the virus with the cellular receptors (ACE2). In this study, the chemicals and detergents having extremely acidic to moderately acidic pH (3.0 to 6.0) and neutral to moderate alkaline pH (7.0 to 10.0) were found to fail to inactivate the AIBV. The AIBV showed clear HA activity with 1% normal cRBC at micro-plate HA test which indicates that all these chemicals failed to inactivate the virus by partial or complete denaturation of the viral enveloped glycoproteins even after exposure to the stipulated period. Findings of the pH sensitivity of the present study highly agree with the findings of other researchers. In their study they found that all the strains of AIBV were found to be resistant at pH 3.0 and most of the strains were found sensitive to pH 11.0 (Otsuki et al., 1979). The infectivity of human coronavirus (SARS CoV-1) was done by using the alkaline (pH > 12.0), or acidic (pH < 3.0) pH containing chemicals or detergent (Darnell et al., 2004).

Results of the pH sensitivity of H9N2 virus indicated that the virus was found highly sensitive at ultra-acidic pH (<3.0) and very strongly alkaline pH (13.0 to 14.0) within few minutes. The highly acidic and alkaline pH containing chemicals might have induced an irreversible conformational change in the enveloped glycoproteins and nucleic acid of these viruses as a result cellular binding and fusion capacity of the hemagglutinin-neuraminidase protein of the virus failed completely and denatured viral nucleic acid as well. Findings of the present study partially agree with the findings of other researchers. Genomic RNA of the H9N2 virus treated with ultra-acidic pH (<3.0) and very strongly alkaline pH (13.0 to 14.0) was failed to multiply chicken embryos and also detected by RT-iiPCR. Shahid et al. (2009) found that the *Orthomyxoviridae* viruses like H5N1 and H9N2 are considered to be sensitive to acidic pH, although their retention of infectivity is dependent on the degree of acidity and strain of virus. The H7N9 viruses lost their infectivity when exposed to the pH 2.0 for 30 min or at pH

3.0 for overnight, while infectivity of the viruses remained unchanged under the pH 4.0 to 12.0 for all contact times. LPAI (H7N2) virus lost 100% of it is infectivity under pH 2.0 for 5 min, but no effect after exposure to the pH 5.0, 7.0, 10.0 or 12.0 for 15 min (Lu et al., 2003). LPAI (H7N3) was unable to retain their infectivity after exposure at pH 1.0, 3.0, 10.0 and 14.0 for 48 h (Muhammad et al., 2001). The pH ranges from 3.0, 5.0, 7.0, 9.0 and 12.0 failed to inactivate the AIV H5N1 viruses after exposure for 5 to 10 min, respectively (Wanaratana et al., 2009).

Newly prepared hand sanitizer was found highly effective against both the viruses of this study. This might be due to presence of 70% ethanol/iso-propanol which has an antiviral activity. Ethanol has been shown to be effective against various enveloped viruses. A concentration of 42.6% (w/w) ethanol was also found to be effective to inactivate the SARS-CoV-1 and MERS coronaviruses, Ebola virus, influenza-A viruses like type H3N2, H1N1 and H3N8 in 30 s of exposure (Siddharta et al., 2017; Kampf et al., 2007). A study result of Lu et al. (2003) indicated that 75% ethanol was found to be efficacious against the two strains of AIVs (H7N9 and H7N2) and other enveloped viruses in 5 minutes. The finding of the present study indicated that 70% ethanol was found to be effective against both the AIBV and H9N2 viruses in 30 s. This finding on the efficacy of hand sanitizer for the inactivation the enveloped RNA viruses of previous studies mostly agree with the findings of the previous studies.

CONCLUSION

Results of the present study using heat and pH treatment of both the enveloped RNA viruses (AIBV and H9N2) clearly indicated that they could easily be inactivated successfully by exposure at high temperature (e.g. 65°C and 70°C) for 15 and 3 min and extremely low (<3.0) and high pH (13.0 to 14.0) for 20 s to 5 min respectively. The AIBV was found highly sensitive to different chemicals, detergents and disinfectants having pH moderately acidic (5.0 to 6.0), neutral to moderate alkaline (7.0 to 10.0) and extremely alkaline pH (13.0 to 14.0) within 20 to 30 s of exposure. On the other hand, bird flu (H9N2) virus was found sensitive to the ultra-acidic (<3.0) and very strongly alkaline pH (13.0 to 14.0) in 5 min. Off the chemicals, both 70 and 40% ethanol/isopropanol could be used for successful inactivation of the RNA viruses' both in-vitro and in-vivo, respectively. Regarding disinfectants, 1% Rok Bleaching solution and 5% Lyzol were found highly effective to destroy both viruses of hospital, toilets, room, conference hall floor and other outside areas within a few minutes. The findings of the present study could be a model for in-vitro as well as in-vivo inactivation of any member virus of the family *coronaviridae* including currently circulating SARS-CoV-2 virus (COVID-19) and other flu viruses responsible for human and animal respiratory diseases and also provides important essential information for public health intervention.

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CONFLICT OF INTEREST

The authors declare that there is no conflicting interest with regards to the publication of this manuscript.

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